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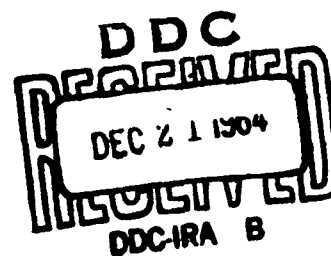
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A NEW METHOD FOR PURIFYING COXIELLA BURNETII BY DEXTRAN SULFATE PRECIPITATION

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A NEW METHOD FOR PURIFYING COXIELLA BURNETII
BY DEXTRAN SULFATE PRECIPITATION

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ABSTRACT

This paper describes a simple method for purifying Coxiella burnetii cultivated in chick embryos. The procedure is based upon the preliminary clarification of infected whole-egg suspensions by precipitating the low-density lipoproteins and other extraneous solids using dextran sulfate, calcium chloride, and sodium chloride solutions, then concentrating the rickettsiae by centrifugation.

I. INTRODUCTION

Methods for purifying and concentrating the rickettsiae, and more specifically, Coxiella burnetii, have been described. A limited number of these reports are cited to illustrate the diversity of procedures employed: use of ether,¹ celite,² differential centrifugation,³ bentonite,⁴ density gradient studies,⁵ differential centrifugation from one molar KCl followed by dimethylsulfoxide and dimethylacetamide.⁶

Although there are many procedures for purifying C. burnetii from chick embryo tissues* methods for efficiently separating the organism from the lipoprotein components and also maintaining viability are limited. The extraction of lipids with ethyl ether⁷ or other nonaqueous organic solvents yielded purified egg suspensions and reduced rickettsial viability. Sakagami and Zilversmit⁸ demonstrated the feasibility of using solutions of dextran sulfate, calcium chloride, and sodium chloride to separate high-density and low-density lipoproteins from dog serum when centrifuged at 4000 x g for 10 minutes. They designated the supernatant fraction as dextran-sulfate-soluble lipoprotein of high density (d 1.063 to 1.210), and the precipitate as dextran-sulfate-precipitable lipoprotein of low density (d 1.063 or less).

The successful isolation of lipoproteins in dog serum by dextran sulfate suggested adaptation of this technique to the purification of C. burnetii from the chick embryo substrate. This communication describes the simple procedure developed for purifying C. burnetii, with particular emphasis given to the relationship between rickettsial viability and lipoprotein purification.

* In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

II. MATERIALS AND METHODS

A. RICKETTSIAL STRAIN AND WORKING STOCK

The AD strain of C. burnetii was employed. The working stock was prepared by harvesting the entire egg contents, except the shell, from embryos dying on the seventh, eighth, and ninth day postinoculation of C. burnetii. The harvest was suspended in one-third its volume with distilled water, homogenized in a Waring Blendor for two minutes, and centrifuged in a Sharples Centrifuge, Laboratory Model, operated at 30,000 rpm, with a flow rate of 500 ml per minute. The resulting supernatant fluid provided the stock suspension that was frozen and stored in a dry-ice chest (-70°C).

B. ASSAY PROCEDURE

Total nitrogen determinations were made by the semi-micro Kjeldahl method;⁹ fat determinations were made by the chloroform extraction methods.¹⁰ Total dry solids were determined with a moisture balance.*

Rickettsial infectivity was estimated by inoculating 250- to 400-gram guinea pigs intraperitoneally with one-ml doses of serial tenfold dilutions made in heart-infusion broth (Difco). Daily rectal temperatures were obtained for each pig for 15 days after inoculation. At 21 days post-inoculation, each guinea pig was bled by cardiac puncture; the blood was processed for serum, which was subsequently inactivated at 56°C for 30 minutes. Each inactivated serum was tested for the presence or absence of specific Q fever antibodies by the standard complement-fixation test procedure. The criteria of infection were: (a) elevation of temperature to 104°F or greater for at least two consecutive days, and (b) the presence of complement-fixing antibody to C. burnetii (in serum taken from inoculated animals). Data on infected pigs (any serum exhibiting a 4+ reaction at 1:4 dilution) for each inoculating dilution were totaled and an endpoint was calculated by the method of Reed and Muench.¹¹ Infectivity data are expressed as log₁₀ guinea pig intraperitoneal infectious doses per ml.

* Cenco Moisture Balance, Central Scientific Co., Chicago, Illinois.

C. PURIFICATION PROCEDURE

Each test day, 400 ml of frozen working stock was thawed and 24 ml of 1.7 per cent NaCl* plus 24 ml of 11 per cent CaCl₂** were added to the stock and lightly agitated by hand, followed by the addition of 80 ml of 5 per cent sodium dextran sulfate*** solution. A flocculent precipitate was separated by centrifugation (Servall SS-4)**** at 2500 rpm for ten minutes (755 x g). The supernatant fraction, designated dextran-soluble lipoprotein, was decanted and stored at 4°C. The precipitate, designated dextran-sulfate-precipitable lipoprotein, was discarded.

To further purify and concentrate the rickettsiae, the supernatant fluid was centrifuged in a refrigerated Servall centrifuge (SS-4) at 8000 rpm for one hour (7710 x g). The rickettsial-rich sediment was recovered and resuspended in distilled water to the original volume of stock as shown in Figure 1.

D. MICROSCOPIC EXAMINATION

Specimens for microscopic examination were prepared by resuspending the final sediment in distilled water as a 1:20 dilution. Smears, stained by the method of Macchiavello,¹² were examined under the oil immersion objective of a light microscope.***** Electron micrographs were made of the suspension with an RCA Model EMU-2 instrument. The grids were prepared by shadow casting with uranium, and pictures were made at an angle of 30 degrees, with a magnification of 20,400.

III. RESULTS

Six representative experiments demonstrating the purification of C. burnetii are shown in Table I. To compare the degree of purification, dry solids, protein nitrogen, and fat analyses data were obtained from the initial suspension (working stock), partially purified supernatant fluid, and the reconstituted sediment. The initial suspension contained

* General Chemical Division, Allied Chemical and Dye Corp., New York, New York.

** J.T. Baker Chemical Co., Phillipsburg, New Jersey.

*** Pharmacia Laboratories, Inc., Sales Office Box 1010, Rochester, Minnesota.

**** Ivan Sorvall, Inc., Norwalk, Connecticut.

***** Bausch & Lomb Model 1-LM-4.

TABLE I. SIX REPRESENTATIVE EXPERIMENTS DEMONSTRATING THE PURIFICATION OF *C. BURNETII*

Experi- ments	<i>C. burnetii</i> Suspensions	Concentration of <i>C. burnetii</i> , Log ₁₀ 10 ⁶ GPIID ₅₀ /ml	Vol, ml	Total Dry Solids, %	Total Nitro- gen, mg/ml	Total Fat, mg/ml	Degree of Purification		
							Contents of Dry Solids Removed from Initial Suspension, %	Contents of Nitrogen Removed from Initial Suspension, %	Contents of Fat Removed from Initial Suspension, %
I	Initial Suspension 66% Whole Egg	2.6	400	17.0	14.133	68.40			
	Partially Purified Supernatant Fluid	1.2	334	4.3	6.702	3.70	78.90	52.58	95.40
	Final Purified Suspension	1.5	400	0.050	0.044	0.030	99.71	99.69	99.96
II	Initial Suspension 66% Whole Egg	3.1	400	17.0	13.398	65.50			
	Partially Purified Supernatant Fluid	2.7	344	4.7	6.516	0.01	76.22	51.17	99.99
	Final Purified Suspension	2.5	400	0.050	0.042	0.02	99.71	99.69	99.99
III	Initial Suspension 66% Whole Egg	4	400	17.0	13.342	65.50			
	Partially Purified Supernatant	3.5	328	4.2	6.643	0.80	79.74	50.21	97.79
	Final Purified Suspension	3.7	400	0.050	0.030	1.20	99.71	99.78	98.17

IV	Initial Suspension 66% Whole Eggs	7.0	400	17.2	13.369	66.80			
	Partially Purified Supernatant Fluid	4.5	328	4.7	6.632	0.96	77.32	51.40	99.77
	Final Purified Suspension	5.5	400	0.06	0.032	0.04	99.76	99.77	99.95
V	Initial Suspension 66% Whole Eggs	1	400	17.0	12.835	81.80			
	Partially Purified Supernatant Fluid	3	334	4.6	6.765	0.08	77.40	47.30	99.91
	Final Purified Suspension	3.5	400	0.04	0.073	0.02	99.77	99.43	99.98
VI	Initial Suspension 66% Whole Eggs	9	400	17.0	13.362	80.80			
	Partially Purified Supernatant Fluid	4.5	336	4.3	6.373	0.80	78.75	52.31	99.01
	Final Purified Suspension	6.0	400	0.05	0.035	0.03	99.71	99.76	99.97

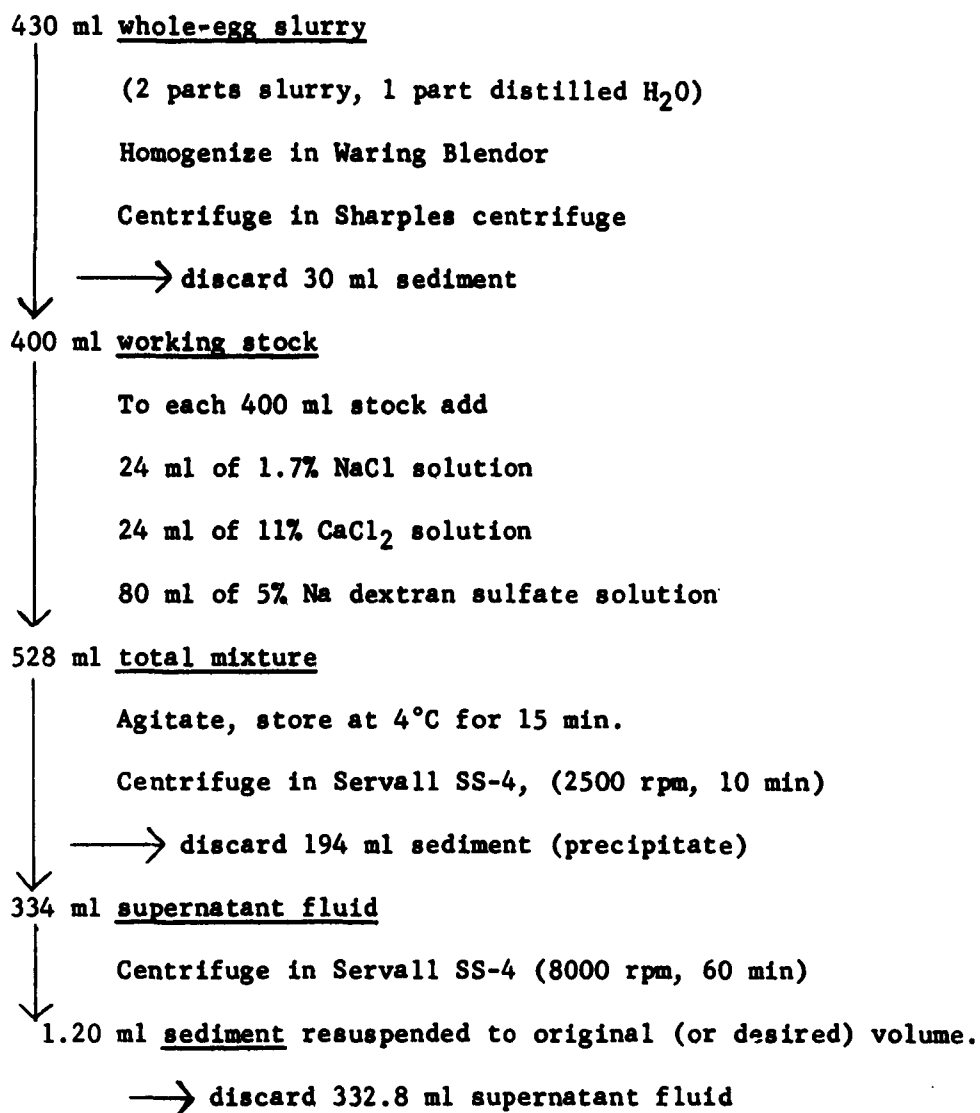


Figure 1. Process flow of dextran sulfate precipitation of whole-egg slurry.

approximately 17 per cent total dry solids, 13.406 mg per ml of nitrogen, and 71.63 mg per ml of fat. The partially purified suspension contained 4.46 per cent total dry solids, 6.605 mg per ml of nitrogen, and 1.06 mg per ml of total fat. The final purified suspension contained 0.05 per cent total dry solids, 0.42 mg per ml of nitrogen, and 0.22 mg per ml of fat. The partially purified suspension, when compared with the initial suspension, contained 78.05 per cent less dry solids, 50.99 per cent less total nitrogen, and 98.81 per cent less fat; moreover, when compared with the initial suspension, the final suspension shows a reduction of 99.73 per cent total dry solids, 99.69 per cent total nitrogen, and 99.84 per cent fats. The final sediment, before resuspension with diluent, constituted approximately 0.3 per cent of the volume of the original suspension or working stock.

Microscopic examination of smears made from the purified suspension and stained by the method of Macchiavello¹² showed that this preparation consisted of relatively pure C. burnetii. An examination of the electron micrograph, Figure 2, indicates that the rickettsiae are virtually free of the insoluble solids of the egg substrate.



Figure 2. Electron Micrograph of Coxiella burnetii. 20,400X

IV. DISCUSSION

A method has been devised for preparing purified suspensions of viable C. burnetii based on the centrifugation of dextran-sulfate-precipitated egg suspensions, followed by another centrifugation to concentrate the rickettsiae from the partially purified suspension. In contrast to previous precipitation methods, particularly the use of nonaqueous organic solvents, it was possible to preferentially separate the organism from the lipoprotein or lipid-like solids without a substantial loss in viability.

To establish an optimum level of precipitation, that is, maximum removal of extraneous solids with minimum rickettsial loss, a series of preliminary titrations, based on the work of Sakagami and Zilversmit,⁸ were made using different concentrations of dextran sulfate, calcium chloride, and sodium chloride solutions. Slight modification of their procedure was necessary in order to obtain the optimum concentration of chemicals required for maximum purification; purification was measured quickly by the amount of dry solids removed from the supernatant fluid. The concentration of each chemical additive is dependent upon the level of solids (total dry) available in the egg suspension. Reducing the solids level of the egg suspension by the addition of various quantities of distilled water changes the requirement for the chemical. For example, maximum precipitation for the initial or working stock suspensions containing 8.5 per cent total dry solids can be achieved with half the concentration of chemicals required for a working stock containing 17 per cent dry solids.

According to Ribí and Hoyer⁵ the density of C. burnetii is 1.11 to 1.18. The success of this method must be due to the preferential precipitation of the low-density lipoproteins (d 1.063 or less) at a neutral pH, leaving the rickettsiae in the supernatant fraction (high-density fraction) that has a density range of 1.063 to 1.21.

LITERATURE CITED

1. Craigie, J. "Application and control of ethyl-ether-water interface effects to the separation of rickettsiae from yolk sac suspensions," Can. J. Res. 23:104-114, 1945.
2. Shepard, C.C., and Topping, N.H. "Preparation of suspensions of rickettsiae from infected yolk sacs without the use of ether," J. Immunol. 55:97-102, 1947.
3. Bovarnick, M.R., and Snyder, J.C. "Respiration of typhus rickettsiae," J. Exptl. Med. 89:561-565, 1949.
4. Polozov, A.I., and Yakovlev. "Method for obtaining purified live cultures of Rickettsia burnetii," Prob. Virol. 6:6:811-814, 1961.
5. Ribi, E., and Hoyer, B.H. "Purification of Q fever rickettsiae by density-gradient sedimentation," J. Immunol. 85:314-318, 1960.
6. Ormsbee, R.A.; Bell, E.J.; and Lackman, D.B. "Antigens of Coxiella burnetii: I. Extraction of antigens with nonaqueous organic solvents," J. Immunol. 88:6:741-749, 1962.
7. Ormsbee, R.A. "A method of purifying Coxiella burnetii and other pathogenic rickettsiae," J. Immunol. 88:1:100-108, 1962.
8. Sakagami, T., and Zilversmit, D.B. "Dextran sulfate precipitation and ultracentrifugation of lipoproteins and hyperscholesterolemic dog serum," J. Lipid Res. 3:111-116, 1962.
9. Clark, E.P. "The determination of nitrogen by the Kjeldahl method," Semi-Micro Quantitative Organic Chemistry, Academic Press, Inc., New York, 1943.
10. Association of Official Agricultural Chemists, "Fats in eggs and egg product," Official and Tentative methods of Analysis of the A.O.A.C., 6th Edition, Assoc. of Agricultural Chemists, Washington, D. C., 1945.
11. Reed, L.J., and Muench, H. "A simple method for estimating fifty per cent endpoints," Am. J. Hyg. 27:3:493-497, 1938.
12. Macchiavello, A. "Estudios sobre tifus exantematico: III. Un nuevo metodo para tenir rickettsia," Revista Chilena Hig. Med. Prev. 1:101-106, 1937.